

Relationship Between Metronidazole Metabolism and Bactericidal Activity

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It has been suggested that the microbicidal effect of metronidazole is mediated by an intermediate in nitro group reduction. We have found that the addition of *Escherichia coli* enhances the lethal effect of metronidazole on *Bacillus fragilis* and suggest that this intermediate may form in one bacteria and kill another. Because acetamide forms during the reduction of metronidazole, we examined the possibility that the same partially reduced intermediate in metronidazole reduction may be both an intermediate in the formation of acetamide and the ultimate reactive form of metronidazole which is responsible for its bactericidal action. Thus, we determined the relationship between bacterial survival and the formation of acetamide when cultures of *B. fragilis*, *Clostridium perfringens*, and *E. coli* were incubated anaerobically in the presence of metronidazole. We found that the log of the early bacterial survival was proportional to the formation of acetamide. The rate of loss of metronidazole was not dependent on the concentration of bacteria in the medium, suggesting that any proposed intermediate formed at a rate which was proportional only to the concentration of metronidazole.

It is widely accepted that reduction of the nitro group of metronidazole is obligatory for biological action of this compound. One type of evidence to support this view is the correlation between the electron affinity of the nitro group in a series of nitroimidazoles and their cytotoxic (1), mutagenic (4), and radiation-sensitizing (2) potencies. Other biological effects also seem to depend on nitro reductase activity. Thus, the sensitivity of nitro reductase activity to oxygen (14) can explain why metronidazole is less bactericidal for aerobic bacteria than for anaerobic bacteria (15) and less cytotoxic for oxygenated tissue culture cells than for cells grown under hypoxic conditions. These biological effects of metronidazole may all be mediated by disruption of deoxyribonucleic acid, a phenomenon that has been observed during metronidazole reduction (8, 11).

N-(2-Hydroxyethyl)-oxamic acid and acetamide form when metronidazole is reduced by either pure or mixed cultures of the intestinal microflora (9, 10) (Fig. 1). Together, these metabolites account for all of the carbon and nitrogen atoms of metronidazole except that of the nitro group. Their origin is compatible with a hydrolytic cleavage between nitrogen atom 1 and carbon atom 2, as well as between nitrogen atom 3 and carbon atom 4 of the imidazole ring. We have suggested that this cleavage occurs on a partially reduced intermediate in metronida-

zole reduction (9). It has also been suggested that a partially reduced intermediate in metronidazole reduction is responsible for the trichomonocidal activity of this drug (7, 12), and it is likely that a partially reduced intermediate is the electrophile, which could explain the weak carcinogenicity of the drug (17). We propose (Fig. 1) the concept of a single partially reduced intermediate which leads either to the two stable metabolites which we have characterized or, alternatively, to a biological action, such as bactericidal activity. Figure 1 shows some (6) but not all (14) of the possible intermediates in nitro group reduction.

It is not clear whether the proposed intermediate is sufficiently stable to leave the bacterium in which it is formed and make a neighboring bacterium nonviable. If the relationship between the bactericidal activity of metronidazole and the formation of a reductive metabolite is clarified, it may help answer this question. It is the purpose of this paper to examine this relationship and thus to infer some of the characteristics that can be attributed to the proposed intermediate.

MATERIALS AND METHODS

Materials. Crystalline metronidazole (mp, 158 to 160°C) was a gift from G. D. Searle and Co., Chicago, Ill. [2-¹⁴C]metronidazole (18.8 mCi/mmol) was a gift from May and Baker Ltd., Dagenham, England. [1-

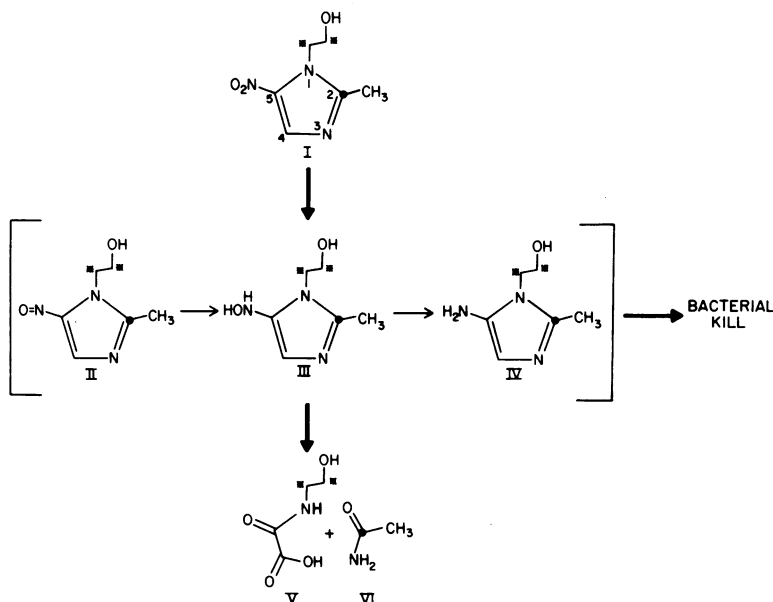


FIG. 1. Scheme of the reduction of metronidazole (compound I) to form acetamide (compound VI) and *N*-(2-hydroxyethyl)-oxamic acid (compound V). The proposed 5-nitroso (compound II), 5-(*N*-hydroxy)-amino (compound III), and 5-amino (compound IV) intermediates are included in the scheme; we suggest that one of these intermediates may be responsible for bactericidal activity. Solid circles indicate the positions of the radiolabel from [2-¹⁴C]metronidazole, and asterisks indicate the positions of the radiolabel from [1',2'-¹⁴C]-metronidazole (9).

¹⁴C]acetamide (3.0 mCi/mmol) was purchased from California Bionuclear Corp., Sun Valley, Calif. All other chemicals were purchased from Aldrich Chemical Co., Metuchen, N.J., Fisher Scientific Co., Boston, Mass., and VWR, Medford, Mass., unless otherwise specified. All bacteria were isolated previously from human feces.

Bacterial incubation conditions. Prereduced anaerobically sterilized Brucella Broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 μ g of hemin per ml was inoculated with *Escherichia coli*, *Bacteroides fragilis*, or *Clostridium perfringens* (9) and incubated overnight at 37°C to obtain cultures in stationary phase. Incubation mixtures which contained both *E. coli* and *B. fragilis* were prepared by mixing equal amounts of the two cultures. Control cultures, which contained only one strain of bacteria in stationary phase, were prepared by making dilutions with an equal volume of medium which had already supported bacterial growth. This medium was prepared by removing the bacteria from a stationary-phase culture by centrifugation at 12,000 $\times g$ for 10 min and then filtering the supernatant liquid through a membrane filter (pore size, 0.45 μ m; Millex; Millipore Corp., Bedford, Mass.). Incubation mixtures contained 10⁶ *E. coli*, *B. fragilis*, or *C. perfringens* cells per ml. When *E. coli* and *B. fragilis* were present together, mixtures contained 10⁶ cells of each strain per ml.

Metronidazole was added to cultures to obtain a final concentration of 100 μ g/ml, and incubations were continued at 37°C in an atmosphere free of oxygen,

which was provided by a V.P.I. anaerobic culture system (Bellco Glass, Inc., Vineland, N.J.), using a gas mixture containing 5% carbon dioxide, 10% hydrogen, and 85% argon. At intervals, samples were removed both for analysis of metabolites and for enumeration of surviving bacteria. To enumerate *C. perfringens* and *B. fragilis*, the samples were serially diluted in sterile 0.9% NaCl, plated onto Brucella Agar supplemented with 5 μ g of hemin per ml, and incubated anaerobically (GasPak; BBL Microbiology Systems, Cockeysville, Md.). Gentamicin (50 μ g/ml) was added to the plates when necessary to prevent the growth of *E. coli*. *E. coli* was enumerated on nutrient agar (Difco Laboratories) which was incubated aerobically.

Analysis of metronidazole and acetamide in bacterial growth media. Samples of the bacterial incubation mixtures were withdrawn at varying times and immediately centrifuged at 550 $\times g$, and the supernatant solutions were filtered (0.45- μ m Millex filter). Portions (2.0 ml) of the filtered incubation mixture were fractionated on an AG 50W-X4 ion-exchange column (20.0 by 1.0 cm; H⁺ form; Bio-Rad Laboratories, Richmond, Calif.). The column was eluted successively with 30.0 ml of water and 50.0 ml of 1 N ammonium hydroxide; 2.0-ml fractions were collected. Thin-layer chromatographic methods (9) indicated that acetamide was the sole radiolabeled compound in fractions 13 to 16 obtained by column chromatography of incubation mixtures containing [2-¹⁴C]metronidazole.

To determine whether acetamide was metabolized

further, [^{14}C]acetamide was added to bacterial incubation mixtures as described above, and samples were removed periodically and filtered; 0.5-ml samples were fractionated on an AG 1-X4 column (0.9 cm by 5 cm; acetate form; Bio-Rad Laboratories). The column was eluted successively with 20.0 ml of water and 20.0 ml of 1 N hydrochloric acid; 2.0-ml fractions were collected. Fractions 2 to 5 contained acetamide, and fractions 14 to 16 contained acetate.

The metronidazole in ethyl acetate extracts of *Brucella* Broth was initially assayed spectrophotometrically by the procedure of Bahnemann et al. (3) and later by high-pressure liquid chromatography, as previously described (10). Both assays were calibrated by using ethyl acetate extracts of standard solutions of metronidazole in *Brucella* Broth. It was also possible to quantify metronidazole on the basis of the radiolabel present in fractions 34 to 36 (where the eluate turned basic) from the Ag 50W-X4 column. The ratio of the amount of metronidazole in the incubation mixture as determined by the radiolabel in fractions 34 to 36 to the amount as determined spectrophotometrically was 1.1 ± 0.2 (mean \pm standard error of the mean), based on five determinations.

Controls containing metronidazole (0.1 mg/ml) were incubated at 37°C in either fresh medium or medium which had already supported bacterial growth (prepared by filtration of a stationary-phase culture through a 0.45- μm Millex filter).

Statistical methods. All linear regressions were determined by the least-squares method. Only after it was determined that the y-intercept was not significantly different from zero were regressions fitted through the origin. All regression coefficients are given with their 95% confidence limits. Regression coefficients were tested for significant difference by using the slope test program of the PROPHET Public Computer System.

RESULTS

Kinetics of bacterial survival in the presence of metronidazole. Survival curves for *E. coli*, *C. perfringens*, and *B. fragilis* with initial concentrations of metronidazole of 0.1 mg/ml are shown in Fig. 2A. Our results confirm those of others in showing a time-dependent decrease in the number of viable bacteria and a greater susceptibility to metronidazole of anaerobic bacteria than aerobic bacteria (5, 16). Figure 2 also shows that the bacterial concentration was in a steady state in the absence of metronidazole under the incubation conditions used. The pH's of the culture media remained unchanged under all incubation conditions used.

The data in Fig. 2A were recalculated by normalizing the bacterial count at each of the early time points to the count at the start of the incubation. The data for these early time points are shown in this form in Fig. 2B. For these time periods bacterial survival may be considered essentially logarithmic. In this formulation the half-lives for *B. fragilis*, *E. coli*, and *C. perfringens* were 0.40, 2.3, and 0.24 h, respectively.

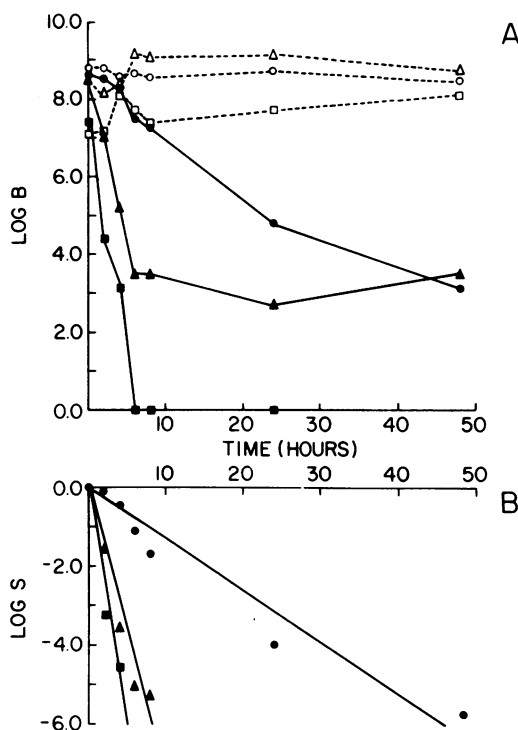


FIG. 2. (A) Time-dependent disappearance of viable bacteria. The number of surviving bacteria (log B) per milliliter is shown for cultures of *E. coli* (○ and ●), *B. fragilis* (△ and ▲), and *C. perfringens* (□ and ■) in stationary phase when the bacteria were incubated at 37°C for 48 h either with (solid symbols) or without (open symbols) metronidazole (0.1 mg/ml). (B) Plot of the log of the surviving fraction of bacteria (log S) during the period when the bacteria displayed in (A) appeared to yield a first-order relationship for bacterial survival.

Enhanced lethal effects of metronidazole on *B. fragilis* in the presence of viable *E. coli* cells. We propose that *B. fragilis* may be more rapidly killed than *E. coli* because it is more susceptible to a reactive intermediate(s) in metronidazole reduction. It is possible that such an intermediate has sufficient stability so that although it is formed in one bacterium, it might diffuse into the medium and interact with another bacterium. Thus, the addition of *E. coli* to a culture of *B. fragilis* might increase the amount of reactive species in the medium and so enhance the lethal effects of metronidazole.

Figure 3 shows the results of an experiment in which the lethal effect of metronidazole on *B. fragilis* was examined in the presence and absence of *E. coli*. It is clear that the addition of *E. coli* resulted in a more rapid killing of *B. fragilis* compared with a control culture which lacked *E. coli*. This experiment was carried out

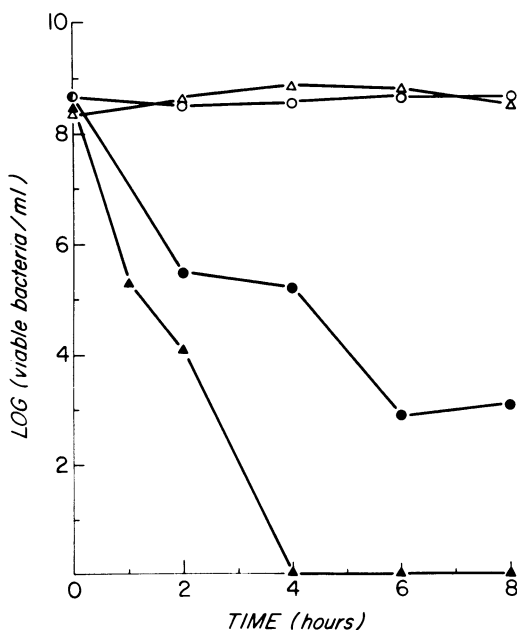


FIG. 3. Effect of metronidazole on the survival of *B. fragilis* in the presence and absence of *E. coli*. *B. fragilis* cells were counted at varying times after initiating incubation in the absence of both *E. coli* and metronidazole (○), in the presence of *E. coli* and absence of metronidazole (△), in the presence of metronidazole and absence of *E. coli* (●), and in the presence of both metronidazole and *E. coli* (▲).

a total of five times, and in every instance the addition of *E. coli* to the medium increased the lethal effect of metronidazole for *B. fragilis*.

It is possible that the observed effect of *E. coli* on the survival of *B. fragilis* was the result of a factor produced by *E. coli* which increased the bactericidal action of metronidazole for *B. fragilis*. This possibility is difficult to exclude, but it was examined (Fig. 4) by adding *B. fragilis* to medium which had already supported the growth of *E. coli*. The enhanced lethal effect of metronidazole on *B. fragilis* did not occur under these circumstances and thus appears to depend on the presence of viable *E. coli* rather than simply on the presence of medium which supported the growth of *E. coli*. Figure 4 also shows that the effect of metronidazole on the viability of *E. coli* was not affected by the presence of viable *B. fragilis* cells. The data are compatible with the possibility that *E. coli* generated a derivative of metronidazole which could be detected by its more pronounced bactericidal effect on *B. fragilis*.

Kinetics of the formation of acetamide from metronidazole. *N*-(2-Hydroxyethyl)-oxamic acid and acetamide are two products of the anaerobic metabolism of metronidazole by *C.*

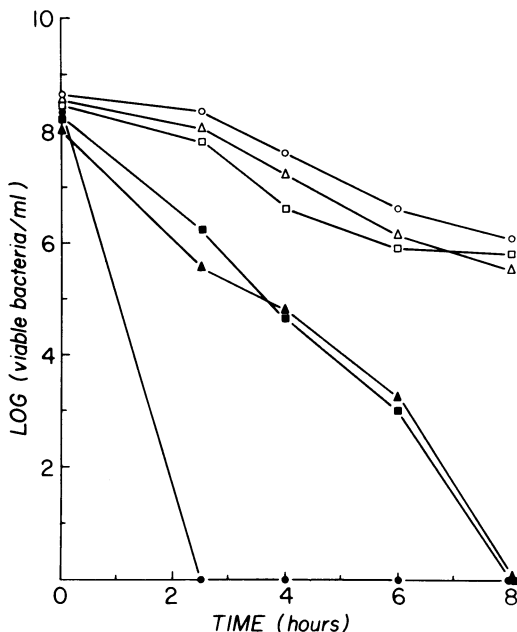


FIG. 4. Effect of different media on the enhancement of the bactericidal action of metronidazole. All incubation mixtures contained metronidazole (0.1 mg/ml). Survival of *B. fragilis* during incubation either with viable *E. coli* (●) or in growth media that had supported either *E. coli* (■) or *B. fragilis* (▲). *E. coli* enumerated during incubation either with viable *B. fragilis* (○) or in growth media that had previously supported either *E. coli* (□) or *B. fragilis* (△).

perfringens (9, 10). We found that *N*-(2-hydroxyethyl)-oxamic acid could not be recovered completely when it was added to cultures of either *E. coli* or *B. fragilis*, whereas acetamide could be completely recovered from cultures of these bacteria and of *C. perfringens* (Table 1). Table 1 also shows that little acetate formed as a result of the hydrolysis of acetamide. Thus, acetamide may be regarded as a stable product of the metabolism of metronidazole in these cultures. Similar experiments indicated that metronidazole was stable when incubated either with fresh medium or with spent media from the culture of all three species of bacteria. Table 2 summarizes the data for the relationship between bacterial survival and the recovery of metronidazole and its metabolite, acetamide. Any proposed model must be in accord with these data.

Relationship between bacterial survival and the loss of metronidazole. Bacterial survival and the metabolism of metronidazole may be related in a number of different ways. In two of the models (Fig. 5), metronidazole (M) undergoes metabolism to form a labile, lethal metab-

TABLE 1. Fate of acetamide added to bacterial cultures^a

Time (h)	% of products recovered in:					
	<i>B. fragilis</i>		<i>E. coli</i>		<i>C. perfringens</i>	
	Acetamide	Acetate	Acetamide	Acetate	Acetamide	Acetate
0	100	0.9	100	1.0	100	1.1
24	96	1.4	94	1.0	101	0.9
48			96	1.1		

^a Acetamide (84.0 μ Ci/mmol; 52 μ g; an amount that reflected a 15% yield of acetamide in terms of the initial metronidazole concentration) was added to 10 ml of culture medium containing 10^8 bacteria per ml, and incubation was continued at 37°C. At the times indicated, 2.0-ml samples of medium were removed for analysis.

TABLE 2. Relationship between bacterial survival and metronidazole metabolism

Time (h)	<i>B. fragilis</i>			<i>E. coli</i>			<i>C. perfringens</i>		
	Log S ^a	Concn of metronidazole metabolized (μ M) ^b	Concn of acetamide formed (μ M)	Log S	Concn of metronidazole metabolized (μ M)	Concn of acetamide formed (μ M)	Log S	Concn of metronidazole metabolized (μ M)	Concn of acetamide formed (μ M)
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	-1.638	19.9	4.7	-0.099	6.4	0.0	-3.2	135.1	89.5
4	-3.505	39.2	19.0	-0.430	45.6	16.4	-4.5	212.3	138.6
6	-5.074	101.8	25.7	-1.113	38.6	14.6			
8	-5.215	63.7	24.6	-1.699	36.3	20.5		304.2	222.3
24		112.3	26.3	-3.94	160.8	69.5		376.2	261.5
48		286.7	64.4	-5.731	222.8	84.8			

^a S, Ratio of the number of viable bacteria at the time shown to the number at the start of the incubation.

^b The initial concentration of metronidazole was 585 μ M in all cultures.

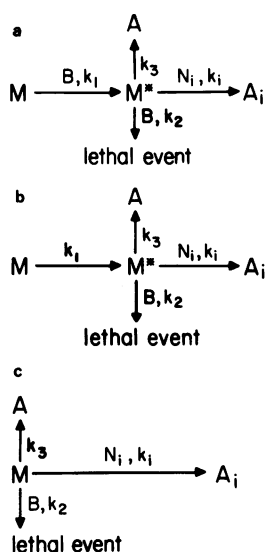


FIG. 5. Three possible models relating the metabolism of metronidazole to bacterial kill. (a) Metronidazole (M) is metabolized by the bacteria (B) at the rate constant k_1 to an active intermediate (M^*), which can react either with bacteria in a lethal manner, with water to yield acetamide (A), or with other chemical species (N_1) in the media to yield other metabolites (A_i). (b) Formation of the active intermediate is not dependent on bacterial concentration. (c) Possible model which has no active intermediate.

olite (M^*), which then either makes a potentially lethal interaction with a bacterium or reacts with another molecule to form one of a number of stable metabolites. We divide the metabolic transformations into the following two kinds: a transformation with water to yield acetamide (A) and transformations with other reactive species present in the culture medium (N_i) to yield other metabolites (A_i).

We can consider two variations of this model. The model in Fig. 5a is based on the assumption that the formation of metabolite M^* is proportional to both the concentration of metronidazole ($[M]$) and the concentration of viable bacteria ($[B]$). According to this model,

$$-\frac{dM}{dt} = k_1[B][M] \quad (1)$$

We can test the applicability of this model by examining the loss of metronidazole as a function of time. As Fig. 6 shows, the loss of metronidazole continues at essentially a constant rate for prolonged periods of time, during which bacterial survival decreases by several logs. Note for example that the rate of loss of metronidazole remained the same at 4 to 6 h in cultures of *B. fragilis* and *C. perfringens* as it was when the concentrations of surviving bacteria were 4 to 5 logs higher (Table 2 and Fig. 2A). Thus, the

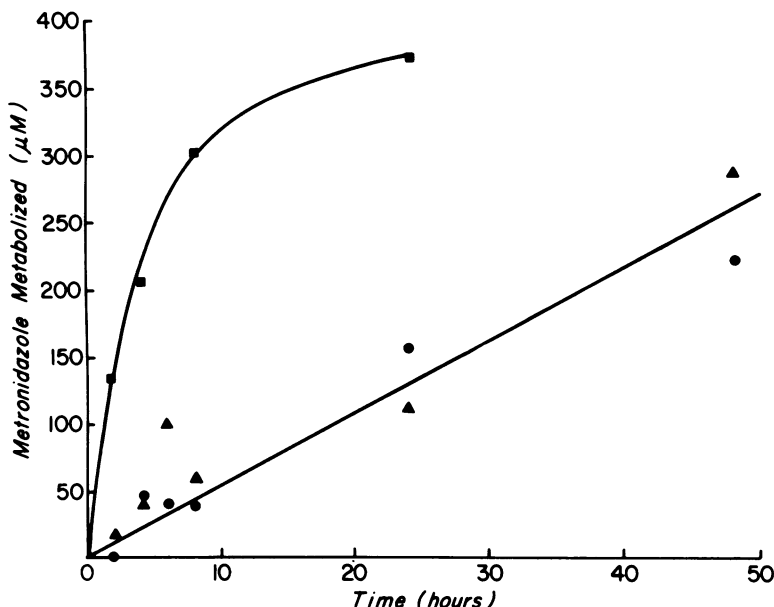


FIG. 6. Time course of metronidazole lost in bacterial incubations. Symbols: Δ , *B. fragilis*; \bullet , *E. coli*; \blacksquare , *C. perfringens*. A single line is used to connect the experimental points obtained for *B. fragilis* and *E. coli*.

model shown in Fig. 5a and its prediction of the relationship in equation 1 must be rejected.

Relationship between bacterial survival and the formation of acetamide. In the model shown in Fig. 5b the loss of metronidazole does not depend on the concentration of bacteria. In this model the loss of bacteria can be expressed as follows:

$$-\frac{d[B]}{dt} = k_2[M^*][B] \quad (2)$$

If, as we have shown, acetamide is stable in the bacterial medium, then it accumulates by the hydrolysis of metabolite M^* according to the following expression:

$$\frac{d[A]}{dt} = k_3[M^*] \quad (3)$$

which is pseudo-first order because of the large excess of water. The expression for metabolite M^* in equation 3 can replace that in equation 2 to yield the following relationship:

$$-\frac{d[B]}{dt} = \frac{k_2}{k_3}[B] \frac{d[A]}{dt} \quad (4)$$

which may be transformed to

$$\frac{d[B]}{[B]} = -\frac{k_2}{k_3}d[A] \quad (5)$$

This expression can be integrated from the start of the logarithmic period of the survival curve (which Fig. 2B indicates is $t = 0$) to a time (t)

during this logarithmic period. At $t = 0$, $[B] = [B]_0$ and $[A] = 0$. At $t = t$, $[B] = [B]_t$ and $[A] = [A]_t$.

$$\int_{[B]_0}^{[B]_t} \frac{d[B]}{[B]} = -\frac{k_2}{k_3} \int_0^{[A]_t} d[A] \quad (6)$$

This yields the expression

$$\ln \frac{[B]_t}{[B]_0} = -\frac{k_2}{k_3} [A]_t \quad (7)$$

which may be converted from natural logarithms to give

$$\log \frac{[B]_t}{[B]_0} = -\frac{k_2}{2.303 k_3} [A]_t \quad (8)$$

Thus, the model shown in Fig. 5b predicts a linear relationship between the log of the fraction of bacteria surviving and the amount of acetamide found in the media.

Figure 7 shows that this relationship was found for each of the three bacterial cultures. These relationships have regression coefficients of -0.20 ± 0.04 , 0.06 ± 0.01 , and -0.033 ± 0.049 and correlation coefficients of 0.98, 0.98, and 0.99 for *B. fragilis*, *E. coli*, and *C. perfringens*, respectively. All three regression coefficients were significantly different when tested for parallelism.

Relationship between the formation of acetamide and the disappearance of metronidazole. Figure 8 shows that the relationship between the amount of metronidazole lost

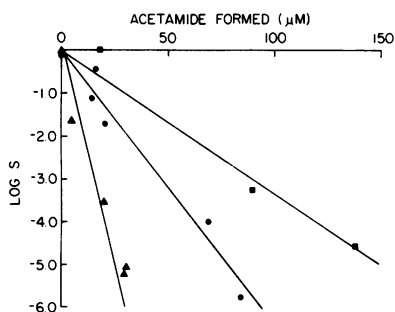


FIG. 7. Relationship between bacterial survival (LOG S) and acetamide formation. Symbols: Δ , *B. fragilis*; \bullet , *E. coli*; \blacksquare , *C. perfringens*.

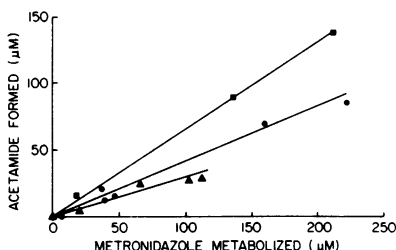


FIG. 8. Relationship between acetamide formation and the amount of metronidazole metabolized. Symbols: Δ , *B. fragilis*; \bullet , *E. coli*; \blacksquare , *C. perfringens*.

and the amount of acetamide formed was essentially linear during the earlier incubation times (those times shown in Fig. 2B). The linear relationships had correlation coefficients of 0.90, 0.99, and 1.0 and regression coefficients of 0.3 ± 0.16 , 0.4 ± 0.03 , and 0.65 ± 0.28 for *B. fragilis*, *E. coli*, and *C. perfringens*, respectively.

This linear relationship is compatible with the model shown in Fig. 5b if it is assumed that the concentrations of metronidazole and metabolite M^* remain proportional and that a relatively small amount of metabolite M^* is consumed by the lethal interaction with bacteria. However, under these circumstances, the data which fit the model shown in Fig. 5b are also consistent with the model shown in Fig. 5c, where metabolite M^* is not required. Thus, our data relating bacterial survival to the formation of acetamide (Fig. 7) are compatible with the two models shown in Fig. 5b and c. The derivation based on the model in Fig. 5c is like that shown for the model in Fig. 5b, except that equations 2 and 3 contain metronidazole rather than metabolite M^* . This modification still yields equation 8, which predicts the relationship of Fig. 7.

DISCUSSION

The presence of viable *E. coli* cells enhances the lethal effect of metronidazole on *B. fragilis*.

This observation is compatible with the possibility that *E. coli* reacts with metronidazole to form a derivative that is stable enough to diffuse out of *E. coli* and kill *B. fragilis*. This derivative could be a radical anion, such as that described by Peterson et al. (14), or it could be an intermediate in the formation of acetamide.

In following up this observation, we explored the possibility that the reactive species is an intermediate in the reductive metabolism of metronidazole which leads to the formation of acetamide. Our kinetic analysis, which examines the relationship between acetamide formation and bacterial survival, neither supports nor eliminates this possibility. The kinetics are compatible with an intermediate whose rate of formation is proportional only to the concentration of metronidazole in the medium and does not depend on the concentration of bacteria. Thus, the postulated common intermediate might be present if its formation were determined, for example, either by the redox potential of the medium or by the presence of enzymes released from nonviable bacteria. A possible bactericidal intermediate in metronidazole reduction, which is also an intermediate in its transformation to acetamide, cannot be further defined by this kinetic argument.

Although it is not necessary to postulate an intermediate to explain our kinetic data, it might be useful to consider the possible implications of our data in terms of such an intermediate. For example, Fig. 7 shows that each bacterial culture has a characteristic relationship between acetamide formation and bacterial survival. If we assume that acetamide formation is dependent (according to equation 3) only on the concentration of metabolite M^* , then the rate of acetamide formation reflects the concentration of metabolite M^* . Under these circumstances the concentration of metabolite M^* would be lowest in cultures of *B. fragilis*, which forms the least acetamide as it is killed, and highest in cultures of *C. perfringens*, which forms the most acetamide as it is killed. A consequence of these assumptions is that *B. fragilis* must be regarded as the most susceptible of the three strains to metabolite M^* , and *C. perfringens* must be regarded as the least susceptible. Thus, bacterial susceptibility to metronidazole might be related to the following two factors: the ability to generate a reactive species and the sensitivity of sites in the bacteria to the actions of that reactive species. Perhaps this will turn out to be a useful concept.

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